FOR THE RECORD

Crystallization of bacteriorhodopsin from bicelle formulations at room temperature

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Abstract

We showed previously that high-quality crystals of bacteriorhodopsin (bR) from *Halobacterium salinarum* can be obtained from bicelle-forming DMPC/CHAPSO mixtures at 37°C. As many membrane proteins are not sufficiently stable for crystallization at this high temperature, we tested whether the bicelle method could be applied at a lower temperature. Here we show that bR can be crystallized at room temperature using two different bicelle-forming compositions: DMPC/CHAPSO and DTPC/CHAPSO. The DTPC/CHAPSO crystals grown at room temperature are essentially identical to the previous, twinned crystals: space group $P2_1$ with unit cell dimensions of a = 44.7 Å, b = 108.7 Å, c = 55.8 Å, β = 113.6°. The room-temperature DMPC/CHAPSO crystals are untwinned, however, and belong to space group $C222_1$ with the following unit cell dimensions: a = 44.7 Å, b = 102.5 Å, c = 128.2 Å. The bR protein packs into almost identical layers in the two crystal forms, but the layers stack differently. The new untwinned crystal form yielded clear density for a previously unresolved CHAPSO molecule inserted between protein subunits within the layers. The ability to grow crystals at room temperature significantly expands the applicability of bicelle crystallization.

Keywords: membrane protein; lipid cubic phase; crystallization method; twinning

Most membrane proteins have been crystallized from a detergent-solubilized form. This approach is popular because crystal trials can be easily performed with the same methods and apparatus used in soluble protein crystallization. Nevertheless, membrane proteins often do not behave well outside of their natural bilayer environment, which precludes the crystallization of many membrane proteins from detergent. To circumvent problems with detergent, Landau and Rosenbusch (1996) introduced a dramatically different technique, lipid cubic phase crystallization. In this method, the membrane protein is crystallized from a bilayer environment. The success of this method demonstrated for the first time that membrane proteins can be crystallized from their natural bilayer. Although a number of membrane proteins have now been crystallized using this method (Kolbe et al. 2000; Luecke et al. 2001; Royant et al. 2001), it has not achieved the popularity of detergent crystallization, in part because of technical difficulties and specialized apparatus needed for handling the extremely viscous lipid cubic phase.

Recently, we developed a bicelle crystallization method (Faham and Bowie 2002) which is somewhat of a compromise between the ease of detergent crystallization and the more protein-friendly lipid cubic phase crystallization. Bicelle lipid/amphiphile mixtures tend to form small bilayer

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Abbreviations: bR, bacteriorhodopsin; DMPC, 1,2 dimyristoyl-sn-glycerol-3-phosphocholine; CHAPSO, 3[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DTPC, 1,2 ditridecanoyl-sn-glycerol-3-phosphocholine; DHPC, 1, 2 dihexanoyl-sn-glycero-3-phosphocholine.

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disks at low temperature, and appear to form a perforated lamellar phase at higher temperature (Nieh et al. 2001). The transition temperature is dependent on the lipid/amphiphile composition and the exact solution conditions. In the perforated lamellar phase, the mixtures form gels, but at lower temperature the mixtures are liquid and can be easily manipulated. Consequently, proteins in bicelles can be handled at low temperature just like proteins in detergent, and crystal trials can be performed in the same manner as detergent crystallization. Thus, the bicelle approach has the advantage of convenience while maintaining the protein in a bilayer-like environment.

We have utilized the bicelle method to obtain high-quality crystals of bacteriorhodopsin (bR), along with a variety of bR mutants (Faham et al. 2004; Yohannan et al. 2004a,b,c). Previously, all bicelle crystallization was performed at 37°C, presumably from the perforated lamellar phase. Many membrane proteins are only marginally stable, however, and may not survive for long periods at 37°C. To make the bicelle method more generally applicable, we assessed whether crystals could be obtained at room temperature. Here we describe two conditions from different lipid/amphiphile compositions that yield high-quality bR crystals at room temperature.

Results

Four different bicelle-forming lipid/detergent compositions were tested for the crystallization of bR at room temperature. These compositions were DMPC/CHAPSO, DTPC/CHAPSO, DMPC/DHPC, and DTPC/DHPC. DTPC was chosen as a possible alternative lipid to DMPC, because it forms a gel at lower temperatures (presumably the perforated lamellar form) than those generally required for formulations using DMPC (Ottiger and Bax 1998, 1999). High-quality bR crystals were obtained with two compositions (DMPC/CHAPSO, and DTPC/CHAPSO).

DTPC/CHAPSO formulation

We identified the initial crystal lead for the DTPC/CHAPSO mixture from a screen of 192 randomly generated conditions. The crystals grew very slowly and even after optimization did not appear before 2-mo incubation. The slow crystal growth rate in DTPC/CHAPSO is probably due to the fact that it gels at room temperature, leading to slower diffusion. PEG is the main precipitant in DTPC/CHAPSO, unlike both crystallization conditions in DMPC/CHAPSO, which are based on salt. Thus, both salts and PEGs should be used in crystal screening trials using the bicelle method. Both planar diamond and rectangular crystal morphologies of bR crystals were observed in DTPC/CHAPSO (Fig. 1A).

The crystals diffracted to ~1.8 Å at the ALS beamline 8.2.2, and a 2.1 Å data set was collected. These crystals belong to the space group $P2_1$ and have the same cell dimensions as bR crystals grown at 37°C in DMPC/CHAPSO (a = 44.7 Å, b = 108.7 Å, c = 55.8 Å, β = 113.6°). Like the prior crystals, the DTPC/CHAPSO crystals were twinned. Using the prior model (1PY6) in molecular replacement, difference maps (F_o–F_o) between the two crystal forms did not show any significant structural changes. Simple rigid body and b-factor refinements yielded an R-factor of 21.9% and an R_{free} of 25.8%. Thus, we conclude that the same crystal form can be obtained from different lipid environments.

DMPC/CHAPSO formulation

bR crystals in DMPC/CHAPSO at room temperature usually grow in 1–2 wks. The crystallization condition is similar to the one used at 37°C, except that at room temperature the use of triethylene glycol as an additive improved the quality of the crystals. The crystals are diamond-shaped (Fig. 1B), similar to the crystals grown at 37°C. X-ray diffraction experiments were performed on five crystals. One crystal indexed in the cell obtained previously. The other four crystals belong to space group $C222_1$ and have unit cell dimensions of a = 44.7 Å, b = 102.5 Å, and c = 128.2 Å. The data can also be indexed in a $P2_1$ cell with the following dimensions: a = 44.7 Å, b = 128.2 Å, c = 56.0 Å, β = 113.6°. Comparing this cell to the cell for the DTPC/CHAPSO crystals suggested that the DMPC/CHAPSO crystals could be packed in a similar manner, but with a b-axis about 20 Å longer.

Molecular replacement was performed for the new crystal form, followed by model building and refinement cycles to a final R-factor of 22.6% and an R_{free} of 25.6% at 2.2 Å resolution (Table 1). In contrast to prior crystal forms, we believe these crystals are untwinned, because the refinement proceeded readily and intensity statistics do not show any sign of twinning (Yeates 1997). As expected, the $P2_1$ and $C222_1$ crystal forms were organized similarly (Fig. 2A,B).

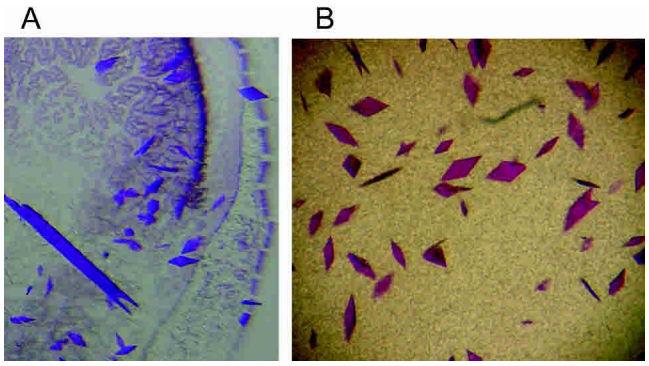

Figure 1. Bacteriorhodopsin crystals. bR crystals grown at room temperature in (*A*) DTPC/CHAPSO and (*B*) DMPC/CHAPSO.

Table 1. *Crystal data collection and refinement statistics*

Data collection	
X-ray source, wavelength (Å)	ALS beamline 8.2.2, 1.1
Space group	$C222_1$
Unit-cell dimensions (Å)	a = 44.66, b = 102.52, c = 128.24
Resolution (Å)	50.0–2.20
Observations, unique-reflections	92,045, 14,746
Completeness (%)	95.6 (87.4)
I/s	10.9 (2.8)
Rmerge	14.2 (32.1)
Refinement statistics	
Resolution (Å)	50.0–2.2
Reflections in working set	13,556
Reflections in test set	1175
R-factor, Rfree	22.58, 25.60
Average B factor (Å^2)	26.7
RMS bonds (Å), angles (degrees)	0.007, 1.1°

Numbers in parentheses refer to the highest-resolution shell.

In both crystals, the protein packs in layers and the packing within each layer remained largely the same. The layers are packed onto each other differently, however. In the $C222_1$ cell there is only one molecule per asymmetric unit, and the layer to layer contacts are mediated by a single loop at residues 162–166. In the $P2_1$ cell there are two molecules per asymmetric unit, and the layer to layer contacts are mediated by a more extensive collection of contacts (A160–A163 contacts A72–A73, B71–B75 contacts B35–B36, and B162–B164 contacts B5).

In the previous crystal form we noticed density between the protein molecules within a layer, but it was not well resolved and could not be identified. In the new, untwinned crystal form, the density is better defined and clearly fits a CHAPSO headgroup (see Fig. 2C–E). Thus, the CHAPSO headgroup mediates crystal contacts, which explains why bR crystallized more readily with the DMPC/CHAPSO and DTPC/CHAPSO formulations than with the DMPC/DHPC and DTPC/DHPC formulations. Although CHAPSO plays an important role mediating crystal contacts in both crystal forms, it is not required for the crystallization of bR in bicelles. We have obtained crystals of bR in DMPC/DHPC bicelles without any additional CHAPSO. These crystals were not optimized sufficiently to yield useful X-ray diffraction.

Discussion

Both of the bR crystal forms that we obtained from bicelles can be described as stacked sheets of two-dimensional (2D) crystals. In both crystal forms, the sheet structures are essentially the same, but they stack differently. This finding, combined with the fact that more surface area is buried

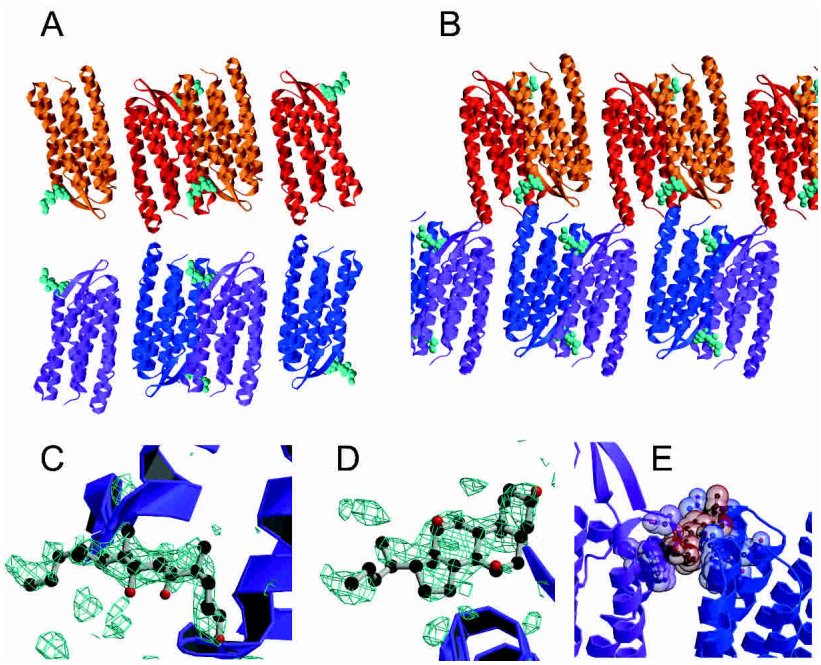

Figure 2. Packing interactions in bR crystals and the role of the CHAPSO molecule. Layer to layer contacts in (*A*) the $P2_1$ crystal form and (*B*) the $C222_1$ crystal form. The locations of CHAPSO molecules are shown in cyan in the CPK model in both *A*,*B*. (*C*) Electron density difference (F_o–F_c), contoured at 2.4 σ, is shown with a side view of the CHAPSO molecule, and (*D*) face view of the CHAPSO molecule. (*E*) Crystal packing interactions of the CHAPSO molecule. The CHAPSO molecule is mediating intra-layer contacts. The CHAPSO is shown in red, and the neighboring bR molecules are in blue and purple.

within sheet layers than between layers, suggests that the intra-sheet contacts are more stable than interactions between sheets. Thus, it seems likely that crystal growth is nucleated by the formation of 2D crystals followed by the facile stacking of these 2D crystals onto each other. There is only a limited requirement for specific contacts between layers. This type of mechanism would explain why twinning is common in the $P2_1$ bR crystal form, and it would explain how the two similar crystal forms could arise.

The ease of use of the bicelle method is based on the ability to control the liquid to gel phase transition. The liquid phase is critical for easily achieving a homogenous sample, while the perforated lamellar gel phase appears to be an ideal phase under which crystal growth can occur. The extended lamella of the gel phase allows protein diffusion within a bilayer environment, while the stacking of the bilayers imparts a partial order that might facilitate crystal growth. The higher viscosity also may be preferred for crystal growth and has been credited with producing better-quality crystals (Thiessen 1994; Vidal et al. 1999). Consistent with the idea that lipid structure facilitates crystal organization, we obtained the same crystal structure in DTPC/CHAPSO and DMPC/CHAPSO, which are both nominally in the gel phase at room temperature and 37°C, respectively. In contrast, when we crystallize in DMPC/CHAPSO from the room temperature liquid phase, a new crystal form is favored with different packing between layers.

The methods described here add to the known lipid mixtures that can facilitate membrane protein crystallization, and we show how the use of different lipids can provide access to both liquid and gel phases at room temperature. Thus, we have increased the repertoire of methods that can be applied to membrane protein crystallization, and have expanded the utility of bicelle crystallization for less-stable membrane proteins.

Materials and methods

DMPC and DTPC were purchased from Avanti Polar Lipids, and CHAPSO was purchased from Sigma-Aldrich. The purple membrane was purified as described by Oesterhelt and Stoeckenius (1974) and was suspended in water to a bR concentration of ~10 mg/mL. In the case of DTPC/CHAPSO, a 40% 3:1 (DTPC:CHAPSO) bicelle mixture was prepared by gentle sonication and then mixed with the protein in a 4:1 (protein:bicelle) ratio. Single crystals grew after 2–3 mo (and sometimes up to 8 mo) using the hanging drop method by mixing 2 μL of the protein/bicelle mixture with 1 μL of a precipitant solution. The precipitant solution contained 100 mM sodium formate pH 4.3, 28.5% PEG 2K, 280 mM ammonium sulfate, and 180 mM hexanediol. About 5 μL of cryo solution was added to the drop before extracting and freezing the crystals. The cryo solution was equivalent to the well solution except that 35% PEG 2K was used. The addition of the cryo solution directly to the crystallization drop also helped to loosen the crystals out of the bicelle gel. In the case of the DMPC/CHAPSO, a 40% 2.8:1 (DMPC:CHAPSO) bicelle mixture was prepared, then mixed with the protein in a 4:1 (protein:bicelle) ratio. Crystals grew in 1–2 wks using the hanging drop method by mixing 4 μL protein/bicelle mixture with 1.5 μL precipitant solution. The precipitant solution contained 2.45 M NaH_2PO_4 pH 3.7, 180 mM hexanediol, and 3.5% triethyleneglycol. In both cases, the protein/bicelle mixture was kept on ice to ensure its fluidity and was homogenized by pipetting up and down. If needed, a quick centrifugation was performed after homogenization to remove any precipitate. Because of the lower gelling temperature, we used extra care in handling the DTPC bicelles by keeping the mixture cold throughout the setup process. Crystal trays were kept in the dark. The crystals were tested for their diffraction quality at the ALS synchrotron beamline 8.2.2. The diffraction data were indexed using DENZO and scaled with SCALEPACK (Otwinowski and Minor 1997). Molecular replacement and refinement were done using CNS (Brunger et al. 1998). 1PY6 was used as an initial model for molecular replacement. Coordinates were deposited with the Protein Data Bank (code 1XJI).

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